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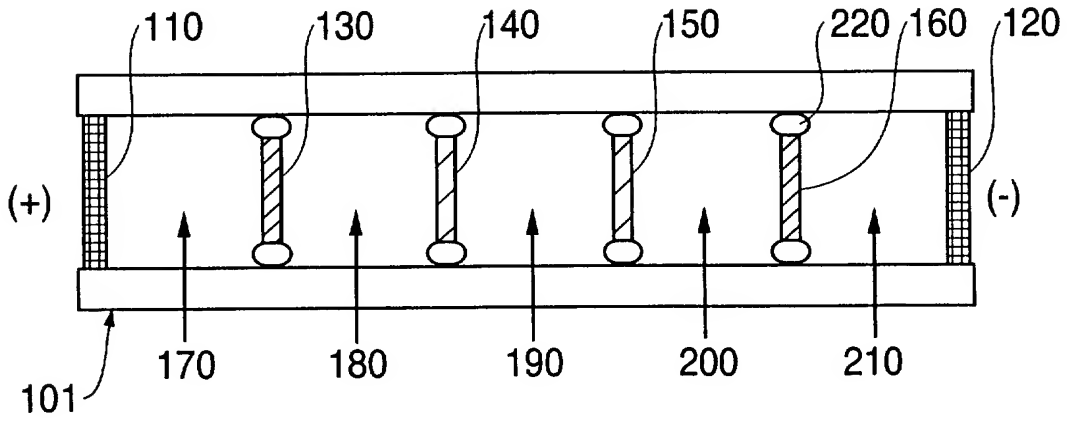
(54) Title: A METHOD AND DEVICE FOR ANALYSIS OF CHARGED MOLECULES BY SOLUTION ISOELECTRIC FOCUSING



(57) Abstract: The invention provides a novel solution isofocusing device and method that can reproducibly fractionate charged molecules into well-defined pools. This approach can be applied to complex charged molecule samples, such as eukaryotic proteome samples where reproducible resolution and quantitation of greater than 10,000 protein components is feasible.

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A METHOD AND DEVICE FOR ANALYSIS OF CHARGED MOLECULES BY SOLUTION ISOELECTRIC FOCUSING

TECHNICAL FIELD OF THE INVENTION

The invention relates to the field of isoelectric focusing of charged molecules, and in particular the isoelectric focusing of proteins.

BACKGROUND OF THE INVENTION

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is currently the only available method for quantitatively comparing changes in protein profiles of cells, tissues or whole organisms (Herbert *et al.* (1997) *in* PROTEOME RESEARCH: NEW FRONTIERS IN FUNCTIONAL GENOMICS (Wilkins *et al.*, eds.), pp. 13-33, Springer, Berlin; Quadroni & James, (1999) *Electrophoresis* **20,** 664-677. The basic method utilizes isoelectric focusing under denaturing conditions in gel tubes or strips that contain either soluble ampholytes (Klose, (1975) *Humangenetik* **26,** 231-243; O'Farrell, (1975) *J. Biol. Chem.* **250,** 4007-4021; Scheele, (1975) *J. Biol. Chem.* **250,** 5375-5385) or immobilines (Bjellqvist *et al.* (1982) *J. Biochem. Biophys. Meth.* **6,** 317-339) followed by a second dimension separation on a conventional SDS PAGE slab gel.

Existing 2D methods are usually adequate for prokaryote proteomes where a maximum of between 1,000 and 3,000 protein spots are expected. In contrast, yeast has over 6,000 genes, *Caenorhabditis elegans* has over 19,000 genes and higher eukaryotes have approximately 100,000 genes. The number of genes that are expressed at any one time in a mammalian cell is not well defined, but estimates of at least 5,000 to 10,000 seem reasonable. In addition, substantial alternative splicing of mRNA's and post-translational modifications of many proteins in higher eukaryotes further increases the total number of protein spots expected (Gooley & Packer (1997) *in* PROTEOME RESEARCH: NEW FRONTIERS IN FUNCTIONAL GENOMICS (Wilkins *et al.*, eds.), pp. 65-91, Springer, Berlin). Current 2D methods lack both adequate resolution and sufficient dynamic range for resolving and detecting the majority of the protein components present in eukaryotic proteomes. Hence, effective analyses of eukaryotic proteomes requires improved protein separation methods capable of resolving and quantitatively detecting the majority of the >10,000 protein components present in whole cell extracts.

One method for resolving an increased number of protein components in eukaryotic proteomes is prefractionation of sample proteins prior to 2D PAGE. Previously reported prefractionation methods include sequential extractions with increasingly stronger solubilization solutions (Molloy *et al.* (1998) *Electrophoresis* **19,** 837-844), subcellular fractionation (Huber *et al.* (1996) *Electrophoresis* **17,** 1734-1740) and selective removal of the most abundant protein components (Lollo *et al.* (1999) *Electrophoresis* **20,** 854-859). Other alternatives include conventional chromatography techniques, such as gel filtration, ion exchange, or affinity chromatography. However, these methods suffer from incomplete separation of proteins between fractions and poor yields are often encountered. Cross contamination of specific proteins between fractionated pools can seriously complicate quantitative analyses and comparisons, since

many proteins appear in more than one fraction and the degree of cross contamination is often highly variable.

Preparative isoelectric focusing as a protein prefractionation procedure was proposed by Bier *et al.* (in: PEPTIDES: STRUCTURE AND BIOLOGICAL FUNCTIONS (Gross & Meienhofer, eds., pp.79-89, Pierce Chemical Co., Rockford, Ill., 1979) and a commercial version called Rotofor was produced by Bio-Rad (Hercules, CA, USA). It is built as a rotating chamber divided into 20 compartments and uses solution isoelectric focusing to fractionate samples. However, this apparatus has no separation barriers and is typically a low resolution technique with relatively large volumes for individual fractions. Righetti *et al.* ((1989) *J. Chromatogr.* **475**, 293-309) described a multi-compartment electrolyser in which each compartment is separated by a polyacrylamide gel membrane with a specific pH. Immobilines are incorporated into the polyacrylamide membranes in the same way that they are to create pH gradients in immobilized pH gradient (IPG) gels. A commercial apparatus, called IsoPrime, incorporating this principle has been marketed (Hoefer Pharmacia, San Francisco, CA). The IsoPrime unit has been developed primarily for large scale purification of individual proteins starting with partially purified preparations, not for fractionation of crude extracts. The unit has large separation chambers connected to peristaltic pumps and external chambers to further expand the volumes of individual fractions (about 30 ml). While the IsoPrime unit can provide high quality separations, its large volume and design make it impractical for prefractionation of samples under denaturing conditions for proteome studies. Similarly, other preparative isofocusing instruments suffer from at least several of the limitations encountered with either the Rotofor or the IsoPrime; specifically: (1) require a large sample volume, (2) produce large volume, dilute fractions that need to be concentrated with attendant losses, (3) exhibit poor resolution, or (4) involve expensive, complex instrumentation.

Prefractionation methods for proteome studies should improve the detection of minor proteins and increase the total number of protein components that can be identified (Quadroni *et al.* 1999; Williams (1999) *Electrophoresis* **20,** 678-688). The ideal prefractionation method would resolve complex protein mixtures such as total extracts of eukaryotic cells or tissues into a small number of well-defined fractions. A small number of fractions is essential, otherwise the already labor-intensive 2D separation becomes prohibitively complex. Yet high resolution is essential to minimize cross-contamination of proteins in adjacent fractions. Commercially available preparative isoelectric focusing (IEF) methods are not suitable for prefractionation, because they typically consume large amounts of sample, result in high protein losses, are much lower resolution than analytical IEF resulting in cross contamination of many proteins between adjacent fractions, and yield very dilute fractions that are incompatible with direct subsequent analysis by 2D gels.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a novel device and method for the separation of charged molecules. These and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention provides a chamber for holding a liquid. The chamber has a first membrane permeable to small ions at a first end, and a second membrane permeable to small ions at a second end and opposite the first end. At least one gel membrane partition is positioned along the chamber to define a plurality of compartments within the chamber, such that each compartment holds a volume of liquid less than 5ml.

Another embodiment of the invention provides a method of separating charged molecules. Charged molecules in solution are added to the compartments of the chamber

described above. A direct current is applied between the first end and the second end of the chamber, whereby the charged molecules are separated.

This invention provides a novel small-scale solution isofocusing device and method that can reproducibly fractionate charged molecules into well-defined pools. This approach can be applied to more complex charged molecule samples, such as eukaryotic proteome samples where reproducible resolution and quantitation of >10,000 protein components is feasible.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 Shows a schematic illustration of one embodiment of a solution isofocusing device, used in the examples.

Fig. 2. Demonstrates the effects of increasing protein amounts on 2D PAGE separations of unfractionated *E. coli* extracts. Different amounts of sample were loaded to pH 4-7L immobilized pH gradient (IPG) strips by rehydration and samples were focused for 60 kVh followed by separation in 10% SDS-gels. Proteins were visualized using Coomassie blue staining. The pH range and location of molecular weight markers are indicated.

Fig. 3. Shows the separation of unfractionated *E. coli* extracts on narrow pH range IPG-based 2D PAGE. Different amounts of sample were loaded to pH 4.8-6.2L IPG strips by rehydration and proteins were focused for 60 kVh followed by separation in 10% SDS-gels. Proteins were visualized using Coomassie blue staining.

Fig. 4. Demonstrates the evaluation of samples prefractionated using an IPG gel. A 1.0 mg aliquot of *E. coli* extract was initially fractionated on a pH 4-7L IPG gel, the focused gel was cut into three sections and proteins were eluted. To check the effectiveness of the initial separation, eluted pools were rerun on pH 4-7L IPG gels for 60 kVh followed by separation in 10% SDS-PAGE gels. Proteins were visualized using Coomassie blue staining. The pH range

of the section from the initial IPG gel that was eluted is indicated above each 2D gel by brackets on the pH scale.

Fig. 5. Shows an evaluation of sample fractionation using solution isofocusing in a representative experiment. Samples from the three separation compartments and the proteins extracted from the separation membranes were evaluated by 2D PAGE after prefractionation of 3 mg of *E. coli* extract using solution isofocusing. One-third of each recovered sample (proportional to 1 mg of original sample) was separated using pH 4-7L IPG gels followed by separation in 10% SDS-gels. Proteins were visualized using Coomassie blue staining.

Fig. 6. Shows a comparison of a composite 2D image from prefractionated samples with the 2D image of an unfractionated *E. coli* extract. (A) Composite 2D protein image produced by cutting and pasting the protein containing sections from the five gels shown in Figure 5. (B) A pH 4-7L 2D gel containing 1.0 mg of unfractionated *E. coli* extract. Proteins were visualized using Coomassie blue staining.

Fig. 7 Demonstrates the effects of IPG strip pH ranges on protein resolution. Replicate pH 5-6 range samples (proportional to 1 mg of unfractionated *E. coli* extract), which were prefractionated using solution isofocusing, were focused using pH 3-10NL, 4-7L and 4.8-6.2L IPG strips, respectively, followed by separation in 10% SDS-gels. Proteins were visualized using Coomassie blue staining (upper panels) and autoradiography (lower panels). Spots were detected and counted using Melanie II software. These values and the effective separation distances for proteins with pI between 5 and 6 are shown above each 2D gel.

Fig. 8. Demonstrates another isofocusing device of the invention. This device contains more separation compartments than the device of Figure 1 in order to cover the full pH range of most proteomes and to increase the total number of spots that should be detected. Each compartment has an access port for improved sample loading and removal.

Fig. 9. Shows a schematic illustrating a method for global analysis of complex proteomes. The specific number of separation compartments and the pH's of the separation membranes can be adjusted to fit different conditions and sample properties. It should be feasible to optimize the pH ranges so that the full resolving capacity of each gel can be utilized. Since each full sized (18 cm X 20 cm) gel should be readily capable of resolving 2,000 to 3000 spots when a high sensitivity detection method is used, the illustrated scheme should be capable of resolving on the order of 10,000 to 15,000 protein spots when complex eukaryotic proteomes are analyzed.

DETAILED DESCRIPTION OF THE INVENTION

Charged molecules, including proteins, can be separated using isoelectric focusing by virtue of their different net charges at a particular pH. The invention provides a novel device and method of isoelectric focusing charged molecules and is especially useful for the separation of a great number of charged molecules. The invention is particularly useful for two-dimensional electrophoretic separations of proteins.

The Chamber

The invention comprises a chamber for holding liquid. As shown in Fig. 1, an embodiment of the invention comprises a chamber (100). A membrane permeable to small ions is located at a first end of the chamber (110). Another membrane permeable to small ions is located at a second end of the chamber (120), opposite of the first end of the chamber. Gel membrane partitions (130, 140, 150, 160) are positioned along the chamber (100) to define a plurality of compartments (170, 180, 190, 200, 210) within the chamber (100). The gel membrane partitions (130, 140, 150, 160) are fitted into the chamber (100) with a seal (220).

Optionally, the gel membrane partition (130) adjacent to the membrane permeable to small ions (110) at the first end of the chamber and the gel membrane partition (160) adjacent

to the membrane permeable to small ions (120) at the second end of the chamber comprise a higher percentage of acrylamide than the remaining gel membrane partitions. In this optional embodiment, the compartment formed by the membrane permeable to small ions at the first end of the chamber (110) and the adjacent gel membrane (130) is a terminal separation compartment (170) and is filled with anode buffer. Further, the compartment formed by the membrane permeable to small ions (120) at the second end of the chamber and the adjacent gel membrane (160) is a terminal separation compartment (210) and is filled with cathode buffer.

A chamber can comprise any suitable material for holding a liquid, for example, teflon or plastic. The chamber comprises a membrane permeable to small ions at a first end of the chamber and at a second end of the chamber, opposite of the first end. The membrane provides protection for the compartments within the chamber and forms a partition. The membrane also prevents proteins with pI's beyond the pH range of the separation compartments from migrating out into the electrophoresis tank. An example of a useful membrane is a dialysis membrane. A dialysis membrane can have a molecular weight cut-off of, for example, at least 1, 5, 10, or 30kDa; however, the use of dialysis membranes with any molecular weight cut-off is contemplated by the invention.

The chamber is divided into separation compartments by gel membrane partitions. Preferably, the gel membrane partitions comprise acrylamide or polyacrylamide. The concentration of acrylamide in gels is stated generally in terms of %T (the total percentage of acrylamide in the gel by weight) and %C (the proportion of the total acrylamide that is accounted for by the crosslinker used). N,N'-methylenebisacrylamide ("bis") is an example of a crosslinker that can be used. Preferably, a gel membrane partition has a large pore size that does not obstruct the movement of charged molecules through or within the gel membrane partition. One of skill in the art can use only routine experimentation to construct a gel membrane partition comprising

a suitable %T and %C. Useful gel membrane partitions can comprise at least 1, 3, 10, 15, or 25%T and at least 1, 5, 8, 15, or 25% C.

Optionally, a gel membrane partition adjacent to the first end of the chamber, *i.e.*, adjacent to the membrane which is permeable to small ions, and the gel membrane partition adjacent to the second end of the chamber, *i.e.*, adjacent to the second membrane permeable to small ions, comprise a higher percentage of acrylamide than any other gel membrane partitions in the chamber. The compartment formed by such a higher percentage acrylamide gel membrane partition and membrane permeable to small ions is termed a terminal compartment.

Preferably, each gel membrane partition comprises a pH different from any other gel membrane partition within a chamber. A gel membrane partition can comprise any pH suitable for the separation of charged molecules. Even more preferably, each gel membrane partition comprises a different pH such that a range of pH's are represented by the gel membrane partitions in the chamber. One of skill in the art can select gel membranes partitions representing a range of pH's that would be useful to fractionate a particular set of charged molecules. Where the gel membrane partitions each comprise a different pH, the gel membrane partitions are preferably arranged from lowest to highest pH within a chamber. The pH of a gel can be adjusted to the desired pH using molecules with a strong buffering capacity, for example, immobilines (Fluka, Milwaukee, WI).

A gel membrane partition can be, for example, at least 0.1 to 5mm thick, preferably a gel membrane partition is at least 0.5 to 2mm thick, even more preferably a gel membrane partition is at least 0.9-1.2 mm thick. The area of a gel membrane partition can be at least 10 mm, 100mm, 250mm, 500mm, or more. A gel membrane partition can comprise any shape that will fit within a chamber, for example, a square or a circle. Any number of gel membrane partitions can be used in the chamber to form any number of compartments within the chamber. For

example, at least 1, 4, 10, 50, 100, or more gel membrane partitions can occur in a chamber to form at least 2, 5, 11, 51, 101, or more compartments.

A gel membrane partition can be mechanically strengthened by embedding a highly porous matrix within the gel membrane partition. For example, non-glass fiber filters or glass fiber filters, such as Whatman GF/D glass fiber filters can be used to strengthen a gel membrane partition.

A gel membrane partition is sealed into a chamber of the invention so that no leakage of liquid or current occurs between the compartments formed by gel membrane partitions. A gel membrane partition can be, for example, fitted into an O-ring or encircled with rubber tubing such that a seal is formed between the O-ring or tubing and the chamber. Alternatively, the gel membrane partition can be cast within a gasket comprising a suitable material, such as a teflon or polypropylene. The gasket comprising a gel membrane partition is fitted into the chamber such that a seal is formed between the gasket and the chamber.

Preferably, the volume of liquid held by each compartment of the chamber is less than 5ml. Even more preferably, the volume of the liquid held by each compartment is less than 4ml, 1ml, or 0.1ml.

Optionally, each compartment of the chamber can comprise an access port, which can be used to insert or extract liquid from each individual compartment. Preferably, the port allows a syringe or pipette tip access to a compartment such that liquid can be added to or removed from each compartment.

Optionally, the chamber can be agitated to provide for movement of liquid within the compartments. The chamber can be agitated by, for example, a magnetic stirrer or by rotating the chamber in a circular fashion.

In a preferred embodiment of the invention an apparatus is provided comprising a chamber, as described above, a tank for electrophoresis, and a power source. The chamber is set into the electrophoresis tank and the tank is filled with anode and cathode buffers. If the chamber comprises terminal separation compartments, these compartments are also filled with anode and cathode buffers. The separation compartments are filled with a sample. A power source is connected to the tank and a voltage is applied.

Methods of Separating Charged Molecules

A chamber as disclosed above can be used to separate charged molecules. The charged molecules migrate under an applied electric current through the chamber until the charged molecule enters a region of the chamber, *i.e.*, a compartment or a gel membrane partition, where the charged molecule has a net charge of zero, *i.e.*, at the charged molecule's isoelectric point. The charged molecules can be a mixture of at least 10, 50, 1,000, 15,000, or more molecules. Preferably, the charged molecules are proteins, even more preferably the charged molecules are prokaryotic or eukaryotic proteomes. A proteome can comprise at least 1,000, 10,000, 15,000, or more protein components.

Any preparation of charged molecules can be separated by the device and method of the invention. Protein samples can be crude or can be partially purified. For example, protein samples can be 25, 50, 75, 90, or 95% purified. A protein sample can also be denatured or in native conformation. Eukaryotic or prokaryotic samples can be can be prepared, for example, by obtaining a population of prokaryotic cells or eukaryotic cells from, for example, cell culture or a tissue sample, and lysing the cells by procedures well known in the art. For example, cells can be pelleted and resuspended in lysis buffer followed by, for example, sonication of the cells. See *e.g.*, Ausubel *et al* (1994) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY,

(Greene Publishing Associates and John Wiley & Sons, New York, NY), and Sambrook *et al.* (1989), MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed. (Cold Spring Harbor Press, Cold Spring Harbor, New York). After sonication, the cell lysate can be centrifuged and treated with SDS. The amount of protein in a sample can be determined using, for example a BCA protein assay (Pierce Chemical Co. Rockford, IL, USA). The samples can be further treated with DNase/RNase (Harper *et al.*, 1995).

A charged molecule sample is solubilized in IPG sample buffer and added to at least one separation compartment. Preferably, the sample in IPG buffer is divided equally by the number of separation compartments and placed into each of the compartments such that each separation compartment is filled. Where the sample is a protein or mixture of proteins, 0.01, 1, 5, 10, or 50 mg of protein can be loaded into each separation compartment. If a chamber comprises terminal separation compartments, these compartments are filled with IEF anode and cathode electrode buffers. These buffers can be made by one of skill in the art and are also commercially available (BioRad).

The filled chamber can be placed into an electrophoresis tank and each of the two compartments of the tank filled with anode and cathode buffers. A power supply can be attached to the electrophoresis tank and voltage applied until the current drops to low levels indicating that equilibrium has been reached as is known in the art. Typically, at least 25,100, 500, 1,000, or 2,000 V can be used for 1,12, 24, or 48 hours. Optionally, the voltage can be adjusted throughout the focusing until the current drops to low levels.

A fractionated sample is removed from each separation compartment. In order to recover a greater portion of the fractionated sample, the surfaces of the gel membranes and the inside walls of the separation compartments can be rinsed with a small amount of a sample buffer and the rinse combined with the fractionated samples. Even greater recovery of the fractionated

sample can be accomplished by removing each gel membrane partition from the chamber and extracting any charged molecules from the gel. These extracted charged molecules can be combined with a fractionated sample, most preferably a fractionated sample that was recovered from a compartment on either side of the particular gel membrane partition that was extracted. Additionally, charged molecules can be recovered from the terminal separation compartments, if present, and combined with the fractionated sample derived from the compartment adjacent to the terminal separation chamber. Preferably, 50, 80, 95, or 100% of the proteins or charged molecules added to the chamber are recovered as fractionated samples.

Once the sample is fractionated it can be, for example, subjected to SDS PAGE. Alternatively, replicate fractionated samples prepared using solution isofocusing can be separated on different pH range IPG strips followed by SDS PAGE. See Fig. 9.

The following are provided of exemplification purposes only and are not intended to limit the scope of the invention described in broad terms above. All references cited in this disclosure are incorporated herein by reference.

EXAMPLES

Example 1 Preparation of Metabolically Radiolabeled *E. coli* Extracts

Metabolically radiolabeled *E. coli* extracts were used in these studies to systematically evaluate protein recoveries. *E. coli* was selected since this relatively simple organism can be readily metabolically radiolabeled to high specific activity to provide sensitive and reliable detection of protein losses. In contrast with chemical labeling methods such as iodination of a portion of the sample, metabolic radiolabeling of the entire sample ensured that the labeling method would not alter the properties of the proteins and that each protein was a homogeneous population of molecules.

E. coli were cultured as previously described (Harper & Speicher, (1995) *in* CURRENT PROTOCOLS IN PROTEIN SCIENCE (Coligan *et al.*, eds.), pp. 6.6.1-6.6.21, John Wiley & Sons Inc., Virginia) with modifications. Briefly, *E. coli* cells were inoculated in Luria broth (LB medium) and incubated at 37 °C with continuous shaking (250-300 rpm) for about 6 h. The LB culture was then inoculated into minimal medium and incubated overnight. When the optical density in the overnight culture reached approximately 1.0 at 550 nm, the culture was diluted 9-fold with methionine- and cysteine- free minimal medium containing 5 µCi/ml of ProMix ^{35}S (Amersham Corp). The cells were cultured until the OD_{550} reached 0.9 - 1.0 to metabolically radiolabel cell proteins to high specific activity.

The *E. coli* were lysed to extract ^{35}S-radiolabeled proteins essentially as previously described by Harper & Speicher (1995). Briefly, the cell culture was harvested by centrifugation at 4000 *g* at 4°C for 20 min and the cell pellet was resuspended in fresh minimal medium and washed once by centrifugation. After the supernatant was discarded, the pellet was resuspended in 5 ml of lysis buffer containing 50 mM NaCl, 50 mM Tris, 5 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.15 mM phenymethylsulfonyl fluoride (PMSF), 1 mM diisopropyl fluorophosphate (DFP), pH 8.0 and was sonicated on ice using a probe-tip sonicator at the lowest power setting for 20 cycles of 15 sec each with a 1 min hold between sonication cycles to prevent overheating. The cell lysate was centrifuged at 48,000 g at 4 °C for 20 min and the supernatant was retained. SDS was added to the *E. coli* extract supernatant to a final concentration of 0.05%. The sample was dialyzed with two buffer changes for about 15 h at 4 °C against the lysis buffer containing 0.05% SDS using 12 kDa cut-off dialysis membranes to remove unincorporated radiolabel. After the amount of protein in the dialyzed sample was determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA), samples in aliquots were stored at -80 °C until required. Immediately before use, samples were thawed, treated with DNase/RNase as

described by Harper *et al.* ((1995) *in* CURRENT PROTOCOLS IN PROTEIN SCIENCE (Coligan *et al.*, eds.), pp. 10.4.1-10.4.36, John Wiley & Sons Inc., Virginia), lyophilized, and dissolved in appropriate IPG sample buffers described in individual experiments.

Example 2: Two-dimensional Electrophoresis

Isofocusing equipment, IPG gels, and relevant reagents were purchased from Amersham Pharmacia Biotech (San Francisco, CA, USA), unless otherwise indicated. Proteins were isofocused using different pH range IPG strips (pH 3-10 NL, 4-7L and 4.8-6.2L, 18 cm length) on the IPGphor™ Isoelectric Focusing System. Narrow pH range IPG gels (pH 4.8-6.2L) were cast in the laboratory using commercial immobilines as detailed in the IPG application manual (LKB Bromma 1990). Immediately prior to IEF, dried IPG strips were rehydrated for 8 h with sample in IPG sample buffer (350 µl) in the ceramic strip holders (1 h without current followed by 7 h at 30 Volts) as described by Görg *et al.* (1999). The IPG sample buffer contained 2 M thiourea, 7 M urea, 0.1 M DTT, 4% CHAPS and 2% IPG-buffer (carrier ampholyte mixture matching the pH range used). After the 8 h rehydration, samples were focused for 1 h each at 500 V, 1000 V, and 2000 V, respectively, followed by 8000 V for a total of 60 kVh.

Immediately prior to loading focused IPG strips on second-dimension gels, the IPG strips were incubated in 10 ml of 50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, 30 mM DTT, pH 6.8, for 10 min, followed by incubation for 10 min in the same solution, except that the DTT was replaced by 2.5% iodoacetamide. The second-dimension SDS-PAGE was performed in 10% acrylamide separating gels prepared as described by Laemmli ((1970) *Nature* **227,** 680-685) using the Iso-Dalt gel format (25 x 20 cm, 1.5 mm thickness) (Pharmacia). The SDS-equilibrated IPG gel was sealed on top of the second-dimension gel using 0.5% agarose containing 50 mM Tris-Cl (pH 6.8), 2% SDS, 30% glycerol and bromophenol blue. SDS gels were run overnight (at 10 °C) until the tracking dye was within 1 cm of the gel bottom. The 2D gels were typically

stained using Coomassie Blue R250. In some experiments, autoradiography was also used to visualize radiolabeled proteins. Briefly, gels were fixed in 10% acetic acid, 30% methanol for 1 h, incubated with EN^{3}HANCE™ autoradiography Enhancer (NEN Products, Boston, MA, USA) for 1 h and dried under vacuum with heat (60-80 °C). The dried gel was exposed to a pre-flashed BioMax MS film using a Transcreen-LE Intensifying Screen (Eastman Kodak Company, Rochester, NY, USA.) at -80 °C for 1.5-7 h. The 2D gels were analyzed using Melanie II 2-D PAGE analysis software (Bio-Rad).

Example 3: Determination of Protein Recoveries

Protein recoveries and losses throughout alternative prefractionation methods were determined using liquid scintillation counting. Any surfaces that contacted samples were extracted with 1% SDS to remove any adsorbed or precipitated proteins. Typically, a small volume of these SDS extracts or sample solutions (5 µl) was mixed with 4.5 ml of Bio-Safe II scintillation cocktail (Research Products International Corp, Mt. Prospect, IL, USA) and radioactivity was counted using a Model 1500 TRI-CARB Liquid Scintillation Analyzer (PACKARD Instrument Company, Downers Grove, IL, USA). The radioactivity left in gels after elution was counted after solubilization using 1 N NaOH at 60 °C for 3 h, followed by neutralization with concentrated HCL and addition of the scintillation cocktail.

Example 4: Evaluation of Maximum Protein Loading Capacity without Sample Prefractionation

Prior to analysis of prefractionation methods, the effects of different protein loads on resolution and spot detection were evaluated using metabolically radiolabeled *E. coli* extracts. The maximum protein loading capacity of any given 2D gel method is an important parameter since higher protein loading capacities should result in the ability to detect lower abundance proteins for any given gel detection and protein identification thresholds.

About 530 protein spots could be detected on Coomassie blue R250 stained gels when 0.5 mg aliquots of the *E. coli* extracts were separated using 18 cm pH 4-7L IPG strips and 18 cm long second dimension gels (Fig. 2). The greatest population of protein components fell within the pH 5-6 range where approximately 240 spots were detected. Although most spots were well separated at the 0.5 mg load, some horizontal streaking of higher molecular weight and basic proteins was observed. Horizontal streaking, which indicates protein precipitation and/or aggregation, decreases the ability to reliably quantitate the proteins involved, can obscure other proteins underlying the streaking and may induce co-precipitation of other proteins. Further increases in protein load did not substantially increase the number of spots detected, but higher loads (2.0 mg) significantly decreased protein resolution due to both increased horizontal streaking and merging major protein spots with their neighbors. These experiments show that about 1.0 mg was the maximum feasible load of unfractionated *E. coli* extracts for this 2D gel system.

The maximum protein loading capacity of narrow pH range IPG strips was also evaluated, since one recommended method for improving 2D gel capacities is to separate replicate aliquots of a total cell extract on parallel narrow pH range gels (Herbert *et al.* (1997) *in* PROTEOME RESEARCH: NEW FRONTIERS IN FUNCTIONAL GENOMICS (Wilkins *et al.*, eds.), pp. 13-33, Springer, Berlin.; Wasinger *et al. Electrophoresis* **18,** 1373-1383.). The results of separating different amounts of unfractionated *E. coli* extract on pH 4.8-6.2 IPG gels are shown in Figure 3. Approximately 320 spots were detected in the pH 5-6 range of the gel when 0.25 mg was loaded and separated and most spots were well resolved with moderate horizontal streaking of some higher molecular weight proteins (>60 kDa). Increasing sample loads (0.5 mg and 1 mg) did not substantially increase the total number of protein spots detected, although more severe horizontal streaking was observed, even at lower molecular weights. As expected, the narrower

pH range gels (pH 4.8-6.2) increased the total number of spots detected by increasing the resolution within this pH range. For example, the total number of spots between pH 5 and 6 were about 320 in the pH 4.8-6.2 gel when 0.5 mg was separated (Fig. 3), but only about 240 spots were detected when the same sample load was separated in a pH 4-7 gel (Fig. 2). However, the narrower pH gels did not increase the maximum sample load capacity when cell extracts were analyzed without prefractionation.

Therefore, a major disadvantage of existing 2D gel methods when applied to proteome analyses of higher eukaryotes is that the maximum sample loading capacity of whole cell or tissue extracts is fairly low, which results in detection of only the most abundant proteins when currently available stains are used (Herbert *et al.* 1997; Williams 1999; Quadroni *et al.* 1999). Increasing the amount of sample above optimal level, *e.g.* 1-2 mg of the *E. coli* extracts in the present study, results in horizontal streaking of many proteins as shown in Figures 2 and 3. Although current IPG-based 2D gels have much higher resolution than alternative separation methods, not all proteins in whole cell extracts can be resolved by a single IPG gel. This incomplete resolution contributes to errors in subsequent quantitation and identification of proteins; that is, a single spot on the gel is frequently not a single protein.

Example 5: Chromatographic Prefractionation of Cell Extracts Prior to 2D PAGE

Several HPLC chromatography methods were initially evaluated as potential prefractionation methods prior to 2D PAGE. HPLC gel filtration in the absence of denaturants resulted in dilute samples, extensive overlap of specific proteins in multiple fractions due in part to the moderate resolution of this chromatographic method and in part to heterogeneous migration of multiple oligomer states. Gel filtration in the presence of denaturants such as urea or SDS primarily separates samples by size similar to the separation that occurs in the SDS gel

dimension. This method does not improve the overall capacity of the separation method since the SDS dimension is typically not the limiting factor in 2D PAGE.

FPLC ion exchange separation in 7 M urea containing buffers with step pH elutions was attempted in an effort to mimic the separation achieved in the isofocusing dimension. The rationale was that simplifying the sample prior to direct loading onto parallel narrow range IPG gels might minimize precipitation and horizontal streaking. Unfortunately, the volumes required to effectively elute proteins at a given pH step were incompatible with direct application to IPG gels, necessitating concentration of the sample. Losses were high, the method was cumbersome, and resolution at the ion exchange step was inadequate with extensive occurrence of proteins in multiple pools, hence severely complicating quantitative comparisons.

Example 6: Prefractionation of Cell Extracts Using Gel-based Isofocusing

The incomplete success of the ion exchange chromatography method suggested that the only separation method with adequate resolution to prevent extensive cross contamination of many proteins between multiple pools would be a high resolution isofocusing method closely analogous to the actual analytical IPG gel method itself. While a same-mode prefractionation approach would not improve overall separation by providing a true third mode of separation or 3D method, it offered the potential of improving capacity and resolution when multiple slightly overlapping narrow pH range gels would be used in parallel.

To evaluate the feasibility of this approach, 1 mg loads of the *E. coli* extracts were initially focused in pH 4-7L IPG gels. After both ends of the gel that were beyond the electrode locations were removed, the remainder of the gel was cut into three parts having pH ranges of 4-5, 5-6, and 6-7, respectively. The gel sections were separated from the supporting plastic film and each section was extracted with three 500 µl volumes of the IPG sample buffer for 1 h per extraction. The three extractions were pooled (~1.5 ml) and concentrated to about 50 µl using

a 10 kDa-cut off CentriCon. The volumes of concentrated samples were adjusted to 350 µl with the IPG sample buffer, loaded to new IPG strips (pH 4-7L), and analyzed by 2D gel to evaluate the feasibility of an IPG gel-based prefractionation approach (Fig. 4). Typically, all the proteins in the pH 6-7 pool were cleanly separated from other fractions. However, some protein spots recovered in the pH 4-5 and pH 5-6 pools were focused at other pH's when the fractionated samples were refocused on the second IPG strips (Fig. 4). This demonstrated that some proteins failed to focus correctly when the 1.0 mg cell extract sample was initially isofocused in the first 4-7L IPG gel. This incorrect focusing is consistent with the observed moderate degree of horizontal streaking of some proteins at 1 mg loads described above (Fig. 2).

Losses of sample proteins during prefractionation using IPG gels were impractically high. Typically, about 16% of the total sample protein was recovered in the strip holder after 1.0 mg of *E. coli* extract was initially isofocused using a pH 4-7L IPG gel with the IPGphor system. The loss of sample proteins at this stage was primarily proteins with pI's outside the pH 4-7 range that run out of the gel and remained in the strip holder. Another 5.5% of total sample protein was lost in the ends of the IPG gel beyond the electrodes, which contained unfocused proteins and was trimmed off after isofocusing, and 3.6% of the total sample was recovered on the IPG gel plastic supporting film. However, the greatest loss (approximate 23%) resulted from the proteins that were not eluted out of the focused IPG gel fractions after the sequential elutions. The total protein recovery in the three eluted fractions was only about 47% of the original sample applied to the IPG gel. These low recovery and incomplete separation of fractions indicated that a scale up of gel-based isofocusing would not be a practical routine method for prefractionating complex samples prior to 2D PAGE.

Example 7: Prefractionation of Samples by Solution Isofocusing

Varying numbers of teflon dialysis compartments with 500 µl volumes (Amika Corp, Columbia, MD, USA) were connected in tandem to construct the novel solution isofocusing device used in the following examples. As shown in Fig. 1 this device has three adjacent separation compartments that were separated by 3% acrylamide gel membrane partitions containing immobilines at the desired pH's. Terminal separation compartments had 10% acrylamide gel membrane partitions with appropriate immobilines. The terminal separation compartments were filled with anode or cathode buffers and were protected from the electrode buffers by 5 kDa dialysis membranes.

Immobiline gels were cast in different concentrations and thicknesses and with several alternative supports for strength. Typically, Whatman GF/D glass fiber filters were imbedded in the gels for mechanical strength using a BioRad mini-gel plate with 1 mm spacers to cast the gels. Gel solutions were prepared as described in Tables 1 and 2. The 25 ml gel solution volume was sufficient for casting two slab gels (1 mm x 7 cm x 10 cm). After the gel was polymerized at 60 °C for about 1.5 hour, 12 mm diameter gel membrane discs were excised from the slab gel using a stainless steel core borer. These gel membrane discs were washed three times with 1 ml of Milli-Q water for 1 h per rinse and soaked in the IPG sample buffer for at least 30 min before use. Unused membranes could be stored in the buffer at 4 °C for up to 3 weeks without affecting the effectiveness of sample prefractionation.

TABLE 1. Preparation of Immobiline Mixtures at Desired pH's

Immobilines	pH 3.5	pH 5.0	pH 6.0	pH 9.5
pK 3.6	299 µl	158 µl		410 µl
pK 4.6	223 µl	863 µl	863 µl	
pK 6.2	157 µl	863 µl	803 µl	
pK 9.3			338 µl	694 µl
Water (up to)	7.5 ml	7.5 ml	7.5 ml	7.5 ml

The mixture should be within 0.05 pH units of the desired pH. If not, the pH should be adjusted using immobilines.

TABLE 2. Preparation of Gel Membranes

	3%T/8%C gel	10%T/8%C gel
Immobiline mixture	7.5 ml	7.5 ml
Acrylamide/Bis (30%T/8%C)	2.5 ml	8.33 ml
Glycerin (87%)	3.45 ml	3.45 ml
Ammonium persulfate (40%)	25 µl	25 µl
TEMED	15 µl	15 µl
Water (up to)	25 ml	25 ml

Example 8: Development of a Solution Isofocusing Method for Sample Prefractionation

A solution isofocusing method was developed and used to separate charged molecules such as a protein extract in tandem small volume liquid-filled compartments separated by thin porous acrylamide gel membranes containing immobilines at specific pH's. A schematic illustration of the prototype device is given in Figure 1. In one embodiment of the invention, teflon dialysis compartments with 500 µl volumes from Amika Corp (Columbia, MD, USA) were used. The unit consists of five compartments and four gel membranes having pH values 3.5, 5.0, 6.0 and 9.5, respectively. Adjacent separation compartments were divided by 1 mm

thick 3% acrylamide gel membranes containing immobilines at the desired pH's. Terminal separation compartments used immobiline gel membranes with 10% acrylamide and these compartments were protected from electrode solutions by dialysis membranes (5 kDa cut-off, Amika Corp). O-rings (12 mm) were used between compartments to assist sealing of gel membranes and compartments by placing an appropriate gel membrane inside an O-ring before the tandem compartments were assembled.

An *E. coli* extract (3 mg) was solubilized in 1.5 ml of IPG sample buffer and divided among the three separation compartments. The terminal separation compartments were filled with BioRad pre-made IEF electrode buffers, 7 mM phosphoric acid (anode) and 20 mM lysine/20 mM arginine (cathode). The assembled compartments were placed into the electrophoresis tank (Amika Corp) and the two compartments of the tank were filled with anode and cathode electrode buffers, respectively. A PS500X power supply (Hoefer Scientific Instruments, San Francisco, CA, USA) was used for focusing the sample. Typically, 100 V was used for 1h (initial ~2-3 mA, final ~1 mA), followed by 200 V for 1 h (initial ~2-3 mA, final ~1 mA), and then 500 V (initial ~3-4 mA) until the current fell to 0 mA (about 1.5 h). After fractionated samples (each ~500 µl) were removed, the surfaces of gel membranes and inside walls of the separation compartments were rinsed with 500 µl of the sample buffer and these rinses were combined with the fractionated samples. The gel membranes were removed and extracted twice with 500 µl sample buffer for 1 h each to elute proteins from the gel matrix. To evaluate the effectiveness of this prefractionation method, one-third of each fractionated sample, which was proportional to 1.0 mg of the original sample, was separated on a pH 4-7L IPG-based 2D PAGE (Fig. 5). These results showed that the cell extracts were well separated into three discrete pools and only a few overlapping spots were found in the pH 3.5-5.0 and 6.0-9.5

fractions. Only a few proteins were eluted from the separation membranes and most of them had pI's close to the membrane pH (± 0.1) (Fig. 5).

A composite image (Fig. 6 A) of the five individual gels shown in Figure 5 can be compared to a 2D gel separation of 1.0 mg *E. coli* extract without prefractionation (Fig. 6 B). The composite image showed that most protein spots in an unfractionated sample were recovered with good yield in prefractionated samples and resolution was improved (Fig. 6 A). Specifically, the total spots in the 2D gel without prefractionation was 545 (Fig. 6 B) compared with 610 spots (Fig. 6 A) in the prefractionated composite image. More importantly, no horizontal streaking of proteins was observed on the composite image with the prefractionated samples (Fig. 6 A), while substantial streaking occurred on the gel with the unfractionated sample (Fig. 6 B).

Total protein recovery of the three solution focused fractions was 65% in these experiments. Another 20% of the total sample proteins was associated with the four gel membranes. About three-quarters of the proteins retained in the membranes could be readily extracted and combined with an adjacent fraction to increase overall yield to about 80%. Finally, about 5% of the total sample was found in the two terminal separation compartments since a small proportion of proteins in the *E. coli* had pI's outside the pH 3.5-9.5 range of the separation compartments used in these experiments. Most of these proteins could be recovered by modifying the experimental design to cover a wider overall pH range if desired. Hence, these experiments demonstrated that this novel device and solution isofocusing technique can rapidly separate complex protein mixtures into a small number of discrete well-defined pools in very high yield (>80%) for subsequent separation on parallel slightly overlapping narrow pH gradient gels.

The solution isofocusing method and device of the invention is much better than eluting proteins from sections of focused IPG gels for prefractionation of samples. Although eluting

proteins from a focused IPG gel can result in reasonably well separated fractions, more than 50% of the sample was lost and not all protein spots in the original sample could be recovered after prefractionation (compare Fig. 4 with Fig. 2). In contrast, prefractionation using the method and device of the invention results in a higher yield (~80%) and more importantly, most protein spots in the original sample can be recovered (Fig. 6). Initial isofocusing in solution minimizes non-ideal behavior of proteins (precipitation/aggregation) encountered if samples are applied to narrow pH IPG gels without prefractionation (Fig. 3). The fractionated proteins exhibit good solubility when applied to narrow pH range IPG gels, which results in better resolution and more spots detected (Figs. 5, 6 A) compared to direct 2D PAGE without prefractionation (Figs. 5, 6, 7). Prefractionation using the method and device of the invention is relatively fast (less than 4 h), requires only a simple, easy-to-use device, and yields well-separated fractions that can be applied directly to subsequent narrow pH IPG strips.

In an initial test of solution isofocusing, 5% gels were used for the separation membranes and 10% gels for the electrode membranes, as suggested for most applications of the IsoPrime method (Righetti *et al.* 1989; Righetti, *et al.* (1990) *J. Chromatogr.* **500**, 681-696; Wenisch *et al.* (1992) *Electrophoresis* **13**, 668-673; Amersham Phamacia biotech 1999). However, when 1 mm 5% gels were used for separation membranes, many higher molecular weight proteins with pI's not equal to the membrane pH precipitated on the gel matrix, thus resulting in a low overall yield (only ~40%) of fractionated samples and poor separation. When the 5% gels were replaced with 3% gels as separation membranes, the yield and separation of fractions were improved, and typically only proteins with pI's equal to the membrane pH were retained in the 3% gel matrix (see Fig. 5). These results suggest that even lower gel densities and thinner gel dimensions can be beneficial if the mechanical strength is maintained to prevent membrane rupture during isofocusing. Also, we stored gel membranes at 4 °C for up to 3 weeks without affecting

performance. Longer term storage is feasible if the membranes are dried and frozen for storage similar to the method used to produce commercial IPG strips. The presence of 2% ampholytes in the solution IEF is advantageous, otherwise current during the electrophoresis is too low to effectively focus the sample. The 2 M thiourea/7 M urea present in the sample buffer is superior to 9 M urea alone for solubilizing sample proteins (Rabilloud *et al.* (1997) *Electrophoresis* 18, 307-316).

The simple solution isofocusing device (Fig. 4) used in this example had three separation compartments and covered the pH 3.5-9.5 range, where most protein spots occurred in the tested sample used. Another device is schematically illustrated in Figure 8. The larger number of compartments and wider pH range will be appropriate for comprehensive or global analyses of complex eukaryotic proteomes. Each compartment has a fill port for more convenient loading and removal of samples. The volumes of separation compartments can be adjusted to fit experimental design such that fractionated sample volumes match the IPG gel rehydration volumes for the number of replicate 2D gels desired. Similarly, the total number of separation compartments and the pH's of divider gel membranes can be altered to fit requirements of specific proteome studies.

Example 9: Effects of Narrow pH Range IPG Gels on Protein Detection and Resolution

A strategy for using the isofocusing device for global analysis of eukaryotic proteomes is illustrated in Figure 9. Prior to 2D gel electrophoresis, a complex sample is fractionated into approximately five pools by solution isofocusing. The pH ranges of each pool should be selected so that similar numbers of spots are obtained on each subsequent 2D gel. For example, in the illustrated scheme the pH 5-6 range has been divided into 0.5 pH units increments since the largest proportion of proteins in eukaryotic proteomes fall in this pH range. As described above proteins with pI's equal to the separation gel membranes are retained in the gel matrix and can

be recovered in reasonable yields by extraction with a small volume of sample buffer. The proteins eluted from the gel membranes can then be combined with the adjacent solution fraction to minimize sample losses at these boundaries. Fractionated samples are then loaded onto narrow pH range gels that are 0.1 pH unit wider than the flanking gel membranes. It should be noted that we used gels that were ± 0.2 pH units wider than the solution pools, but the accuracy of the pI's from these pools was sufficiently precise that ± 0.2 pH units wider gels can be adequate. Parallel SDS-gels are used for second-dimension separation electrophoresis. The ability to increase the protein load per narrow pH range gel using prefractionated samples compared with unfractionated samples will improve the dynamic range of the global proteome analysis by permitting detection of less abundant spots for any given detection method and by increasing separation distances between minor components and major components. In addition, the prefractionation step more efficiently utilizes samples available in limited amounts compared with analysis of unfractionated samples on narrow pH range gels. Since each narrow pH range gel has the capacity to resolve approximate 2,000 to 3,000 protein components, the scheme showed here with separation of a sample into five fractions to be loaded onto five overlapping narrow pH range gels results in a robust method for reliably detecting at least 10,000-15,000 protein components.

The feasibility of using narrow pH gradient gels with prefractionated samples described above was evaluated. Replicate fractionated samples (pH 5-6) prepared using solution isofocusing were separated on different pH range IPG strips (pH 3-10NL, 4-7L and 4.8-6.2L) followed by SDS-PAGE (Fig. 7). The protein spots in the 2D gels were found only within the pH 5 to 6 range regardless of IPG gel pH ranges or detection methods used. These results verify that the components in the pH 5-6 fractionated sample were well resolved from other pH fractions with no detectable cross-contamination of proteins after the prefractionation step even

when the more sensitive autoradiography detection method was used (Fig. 7). The advantage of using narrow pH gradient gels with sample prefractionation was clearly demonstrated by the improved resolution of a fractionated pool on narrow pH range gels. Specifically, about twice as many protein spots were detected on an 18 cm pH 4.8-6.2 L gel compared with a pH 3-10NL gel with either Coomassie Blue detection (355 spots vs. 187 spots) or the more sensitive autoradiographic detection (543 spots vs. 281 spots).

The advantages of prefractionation are further illustrated by comparing the 2D gels using pH 4.8-6.2L IPG strips to the 2D PAGE analysis of unfractionated samples using the same narrow pH range (Fig. 3). No horizontal streaking of proteins was observed on the 2D gel with prefractionation (Fig. 7), but substantial horizontal streaking occurred on 2D gels loaded with unfractionated samples (Fig. 3). Apparently, prefractionation using solution isofocusing eliminates interfering components with pI's beyond the pH range of a given narrow IPG gel which otherwise may precipitate or aggregate in the gel. Hence, the prefractionation step results in overall load capacity increases over alternative 2D PAGE methods such as direct use of parallel narrow pH range gels without prefractionation. Increased sample loads without precipitation or aggregation using this system should improve reliability of quantitative comparisons, increase the number of spots that can be resolved and allow detection of lower abundance spots. Finally, prefractionation more effectively utilizes samples that are available in limited amounts compared with replicate application of unfractionated samples to multiple different narrow pH range gels.

5

CLAIMS

We claim:

10 1. A chamber for holding a liquid, the chamber having a first membrane permeable to small ions at a first end, and a second membrane permeable to small ions at a second end and opposite the first end; and at least one gel membrane partition positioned along the chamber to define a plurality of compartments within the chamber, such that each compartment holds a volume of liquid less than 5ml.

15 2. The chamber of claim 1 wherein the gel membrane partition comprises acrylamide.

3. The chamber of claim 2 wherein the gel membrane partition adjacent to the first membrane permeable to small ions at the first end of the chamber and gel membrane partition adjacent to the second membrane permeable to small ions at the second end of

20 the chamber comprise a higher percentage of acrylamide than the remaining gel membrane partitions.

4. The chamber of claim 1 wherein each gel membrane partition comprises a different pH.

5. The chamber of claim 1 comprising at least 4 gel membrane partitions .

6. The chamber of claim 1 wherein the area of a gel membrane partition is less than 100 mm.

7. The chamber of claim 1 wherein the chamber further comprises an access port for each compartment.

8. The chamber of claim 1 wherein each compartment holds a volume of liquid less than 4 ml.

9. The chamber of claim 1 wherein each compartment holds a volume of liquid less than 1 ml.

10. An apparatus for separating charged molecules comprising:

the chamber of claim 1;

an electrophoresis tank; and

a power source.

11. A method of separating charged molecules comprising the steps of:

adding charged molecules in solution to the compartments of the chamber of claim 1; and

applying a direct current between the first end and the second end of the chamber, whereby the charged molecules are separated.

12. The method of claim 11 wherein the charged molecules are separated according to their isoelectric points.

13. The method of claim 11 wherein anode buffer in placed in the compartment of the chamber of claim 1 having the first membrane permeable to small ions as a partition; and

cathode buffer is placed in the compartment of the chamber of claim 1 having the second membrane permeable to small ions as a partition;

14. The method of claim 11 wherein the charged molecules are proteins.

15. The method of claim 14 wherein at least 50 proteins are added.

16. The method of claim 14 wherein at least 10,000 proteins are added.

17. The method of claim 14 wherein the proteins comprise a proteome.

18. A method of two-dimensional electrophoresis comprising the steps of:

(a) separating a protein sample by the method of claim 11; and

(b) subjecting the separated protein sample of step (a) to SDS gel electrophoresis.

19. A method of two-dimensional electrophoresis comprising the steps of:

(a) separating a protein sample by the method of claim 11;

(b) further separating protein samples of step (a) using immobilized pH gradient gels; and

(c) subjecting the separated protein sample of step (b) to SDS gel electrophoresis.

FIG. 1

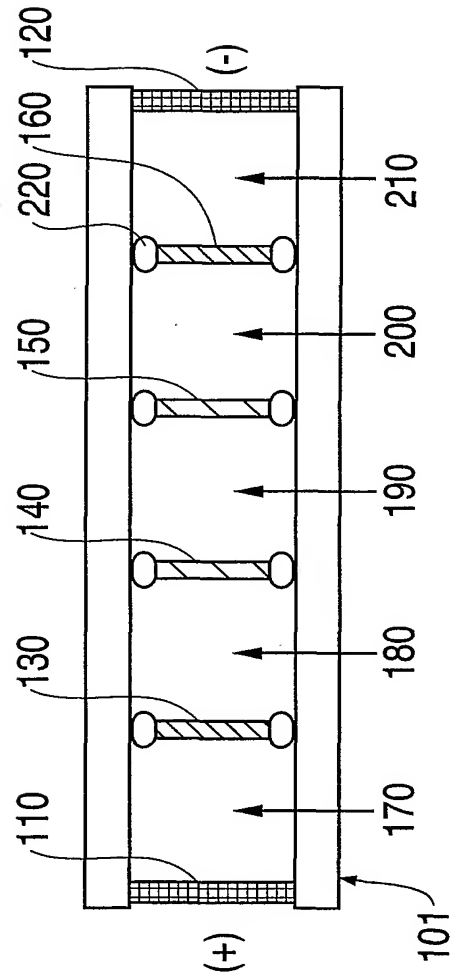


FIG. 2

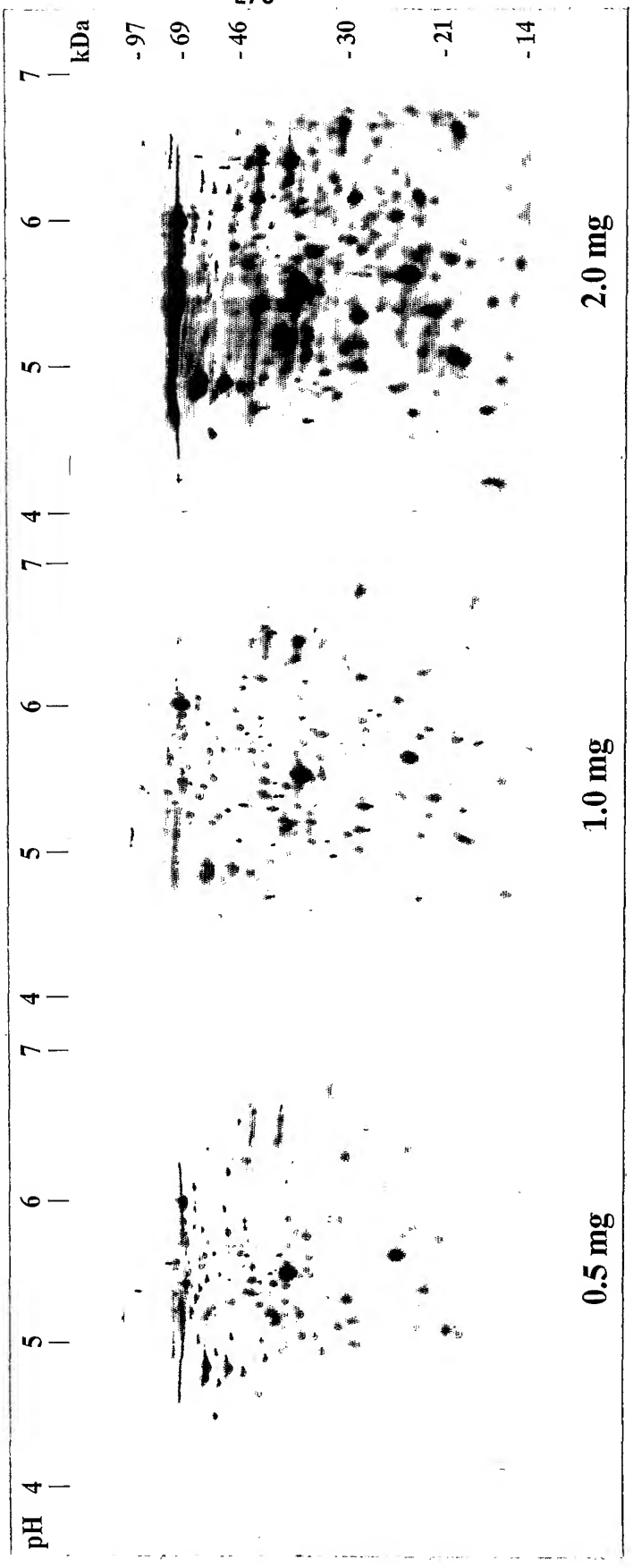


FIG. 3

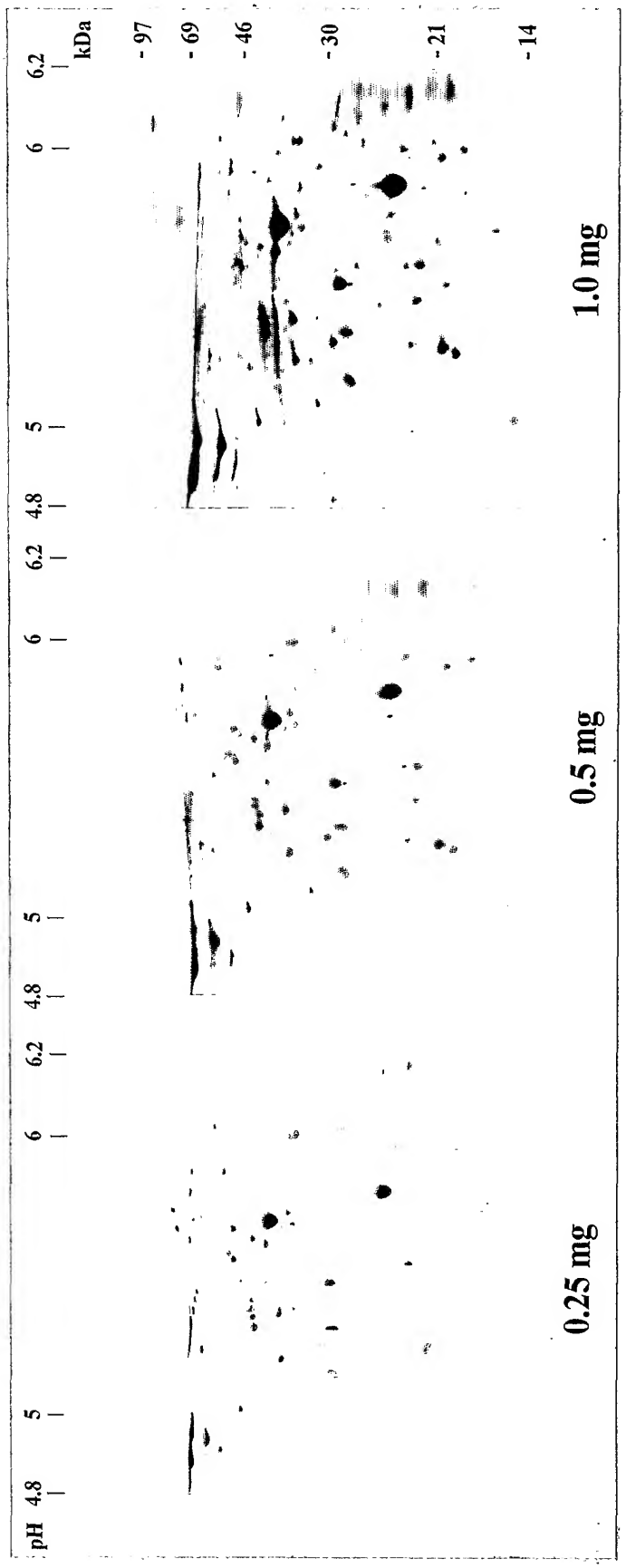
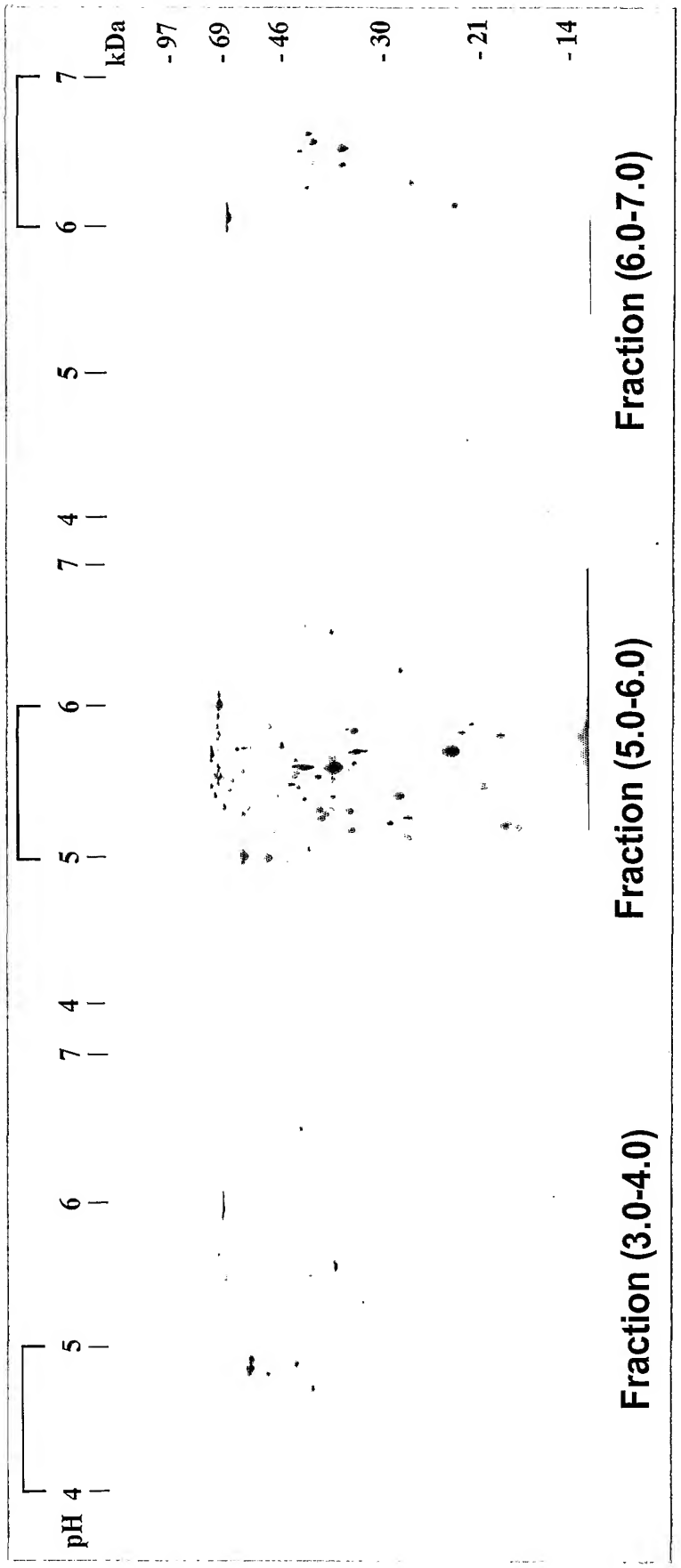


FIG. 4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6
7
4
5
6
7
4
5
6
7
kDa
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-69
-46
-30
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-14
Fraction (3.0-4.0)
Fraction (5.0-6.0)
Fraction (6.0-7.0)

FIG. 5

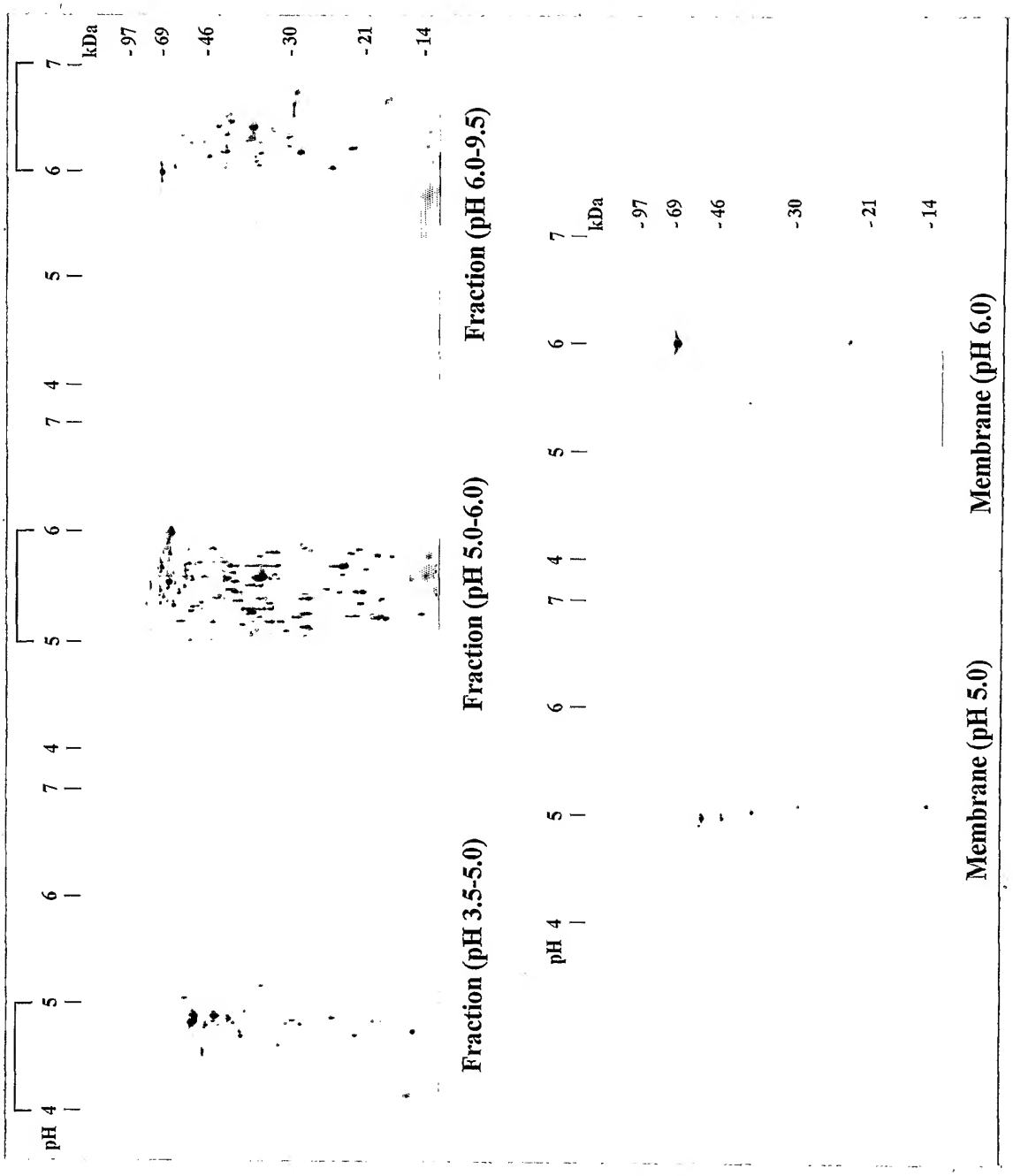

pH 4 5 6 7
kDa
-97
-69
-46
-30
-21
-14
Fraction (pH 3.5-5.0)
Fraction (pH 5.0-6.0)
Fraction (pH 6.0-9.5)
Membrane (pH 5.0)
Membrane (pH 6.0)

FIG. 6

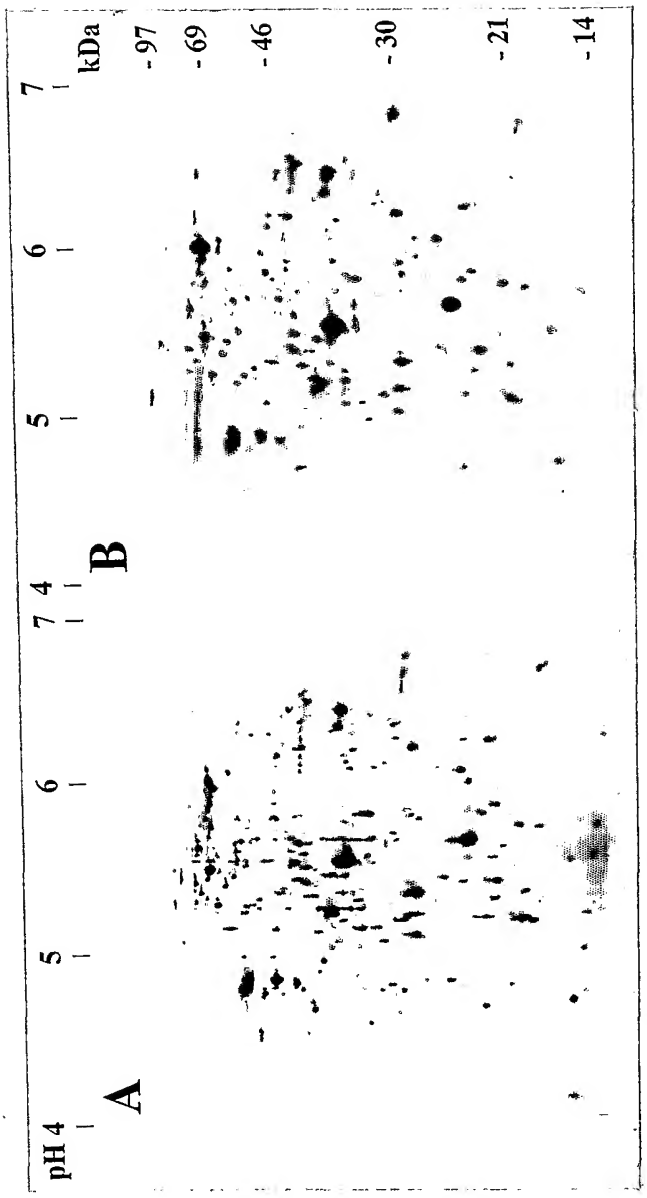
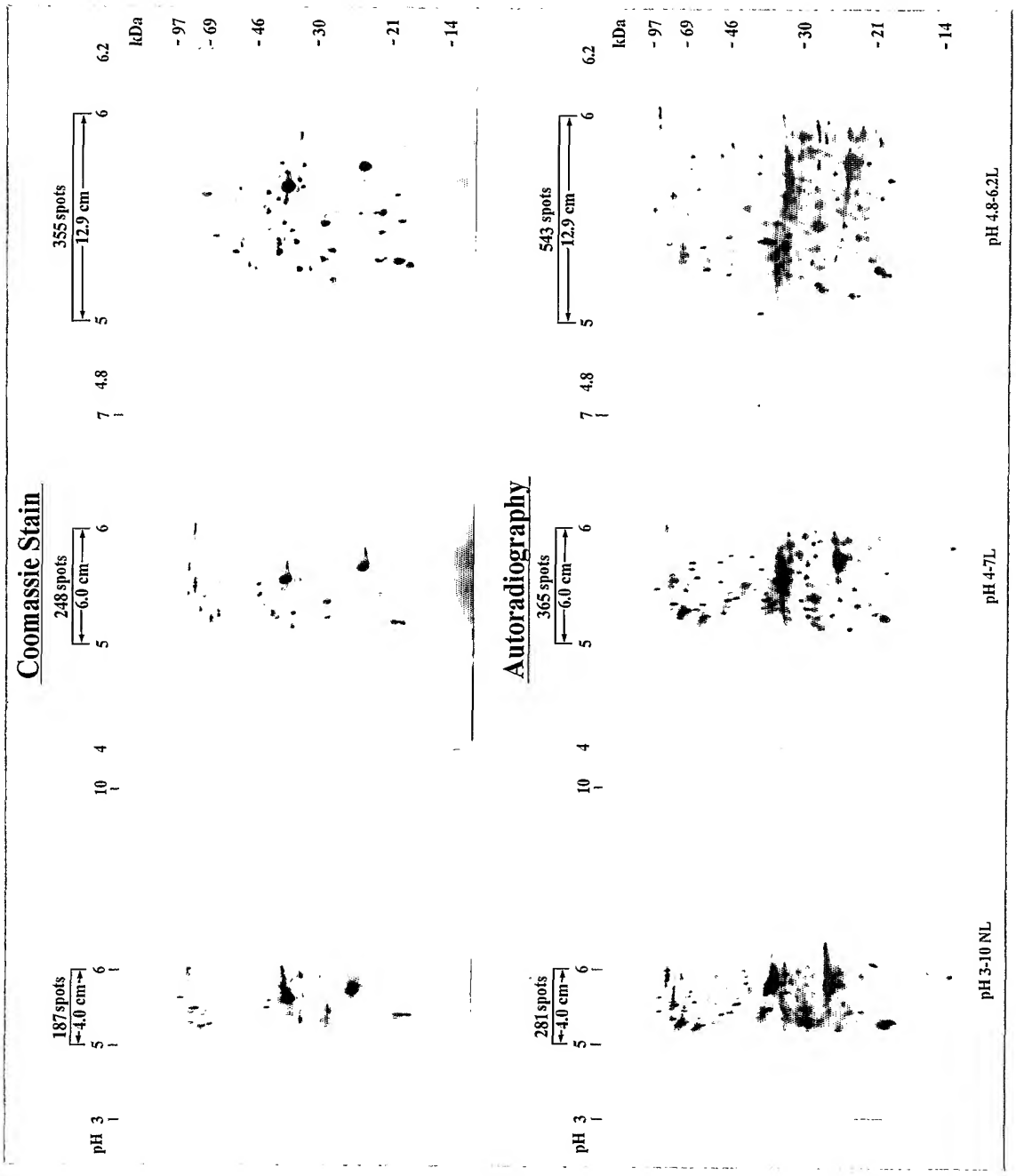


FIG. 7


Coomassie Stain
Autoradiography
187 spots
4.0 cm
248 spots
6.0 cm
355 spots
12.9 cm
281 spots
4.0 cm
365 spots
6.0 cm
543 spots
12.9 cm
pH 3
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7
4.8
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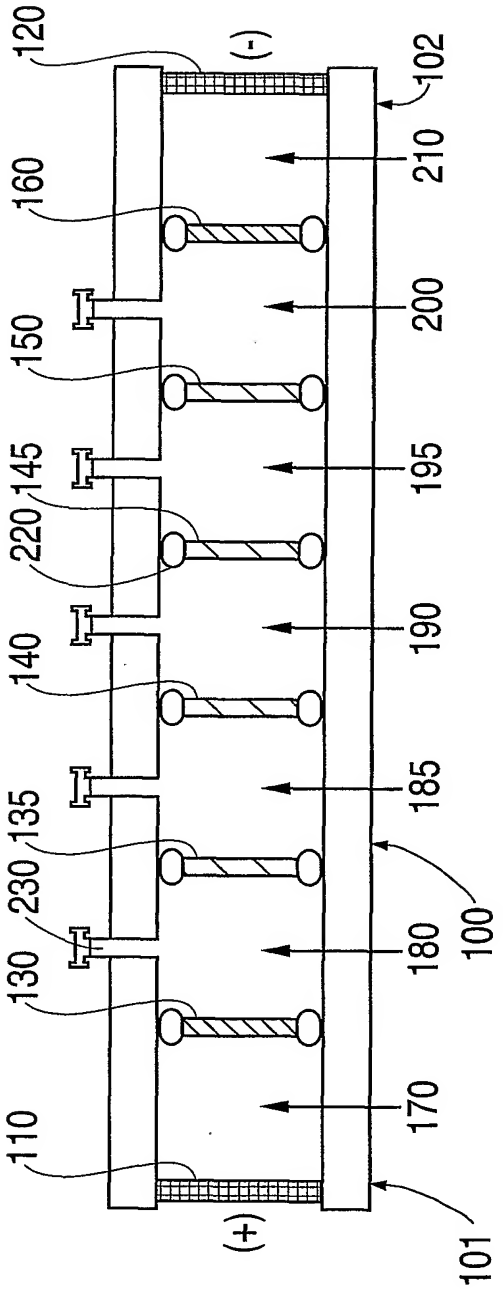
30
21
14
pH 3-10 NL
pH 4-7L
pH 4.8-6.2L

FIG. 8


120
(-)
102
210
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200
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(+)
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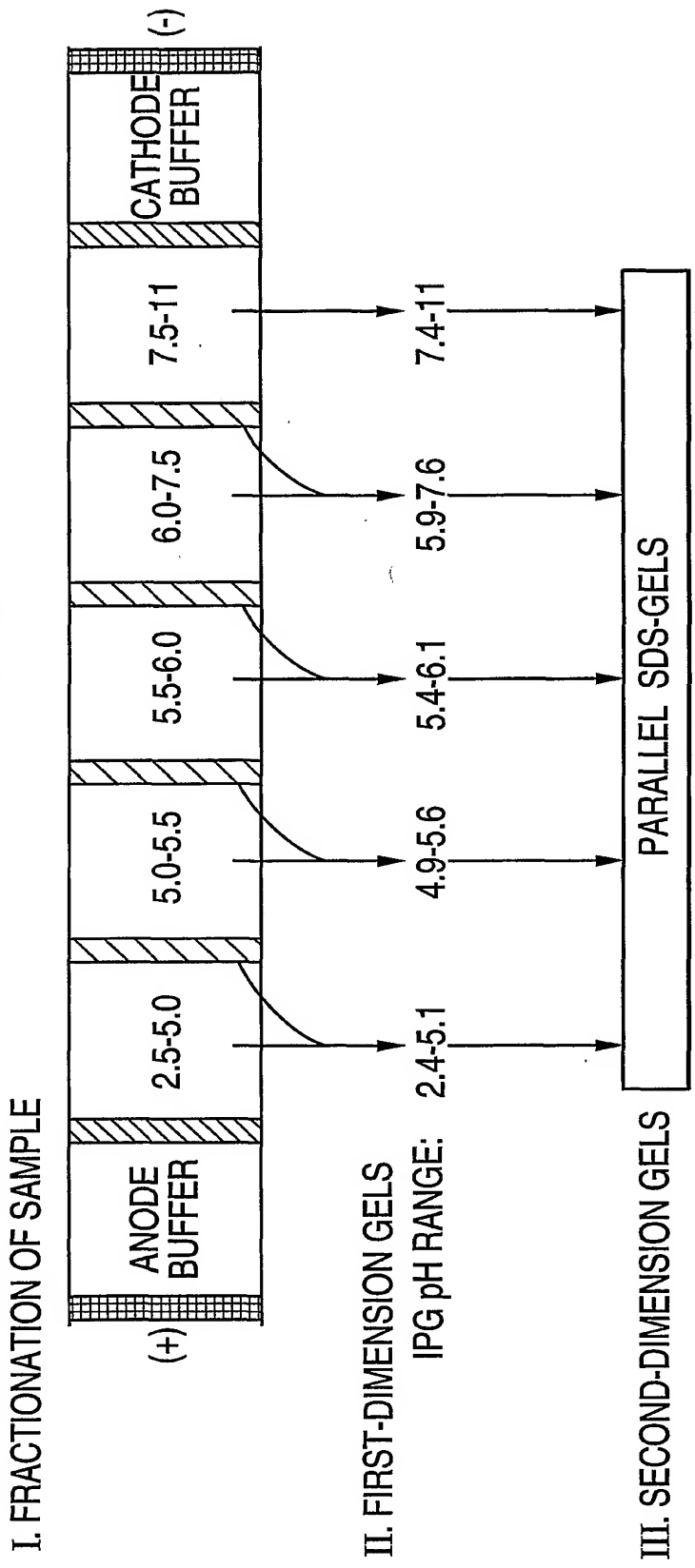
FIG. 9


I. FRACTIONATION OF SAMPLE
(+)
ANODE BUFFER
2.5-5.0
5.0-5.5
5.5-6.0
6.0-7.5
7.5-11
CATHODE BUFFER
(-)
II. FIRST-DIMENSION GELS
IPG pH RANGE:
2.4-5.1
4.9-5.6
5.4-6.1
5.9-7.6
7.4-11
III. SECOND-DIMENSION GELS
PARALLEL SDS-GELS